

Elsevier Editorial System(tm) for Molecular Immunology

Manuscript Draft

Manuscript Number:

Title: THE T-CELL RECEPTOR BETA CHAIN FROM SEA BREAM (*Sparus aurata*): MOLECULAR CLONING, EXPRESSION AND MODELLING OF THE COMPLEXES WITH CLASS I MHC

Article Type: Full Length Article

Section/Category:

Keywords: T-cell antigen receptor, TcRb, *Sparus aurata*, real time PCR, 3D structure, class I MHC, TcRb/MHC complexes

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Manuscript Region of Origin:

Abstract:

Dear Prof. M.R. Daha,

I am submitting you the paper: THE T-CELL RECEPTOR BETA CHAIN FROM SEA BREAM (*Sparus aurata*): MOLECULAR CLONING, EXPRESSION AND 3D STRUCTURAL ANALYSIS OF THE COMPLEXES WITH CLASS I MHC, author team: Elisa Randelli, Viviana Scala, Daniela Casani, Susan Costantini, Angelo Facchiano, Massimo Mazzini, Giuseppe Scapigliati, Francesco Buonocore for the publication on Molecular Immunology.

This paper is related to fish immunology and is focused on a molecule fundamental for adaptive immunity and for sea bream responses to virus and parasites.

Best regards

Dr. Francesco Buonocore, Ph.D  
Univ. of Tuscia  
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**ABSTRACT**

The T-cell antigen receptor (TcR) is a fundamental mediator of the adaptive immune responses, since TCR $_{\alpha\beta}$  molecules on T-cells recognize foreign structures (peptides derived from processed antigens) bound to major histocompatibility complex (MHC) antigens on another cell. In the present study we report the cDNA sequences of six TcR $\beta$  chains from sea bream (*Sparus aurata*), a fish of high economical impact in South Mediterranean aquaculture. Domains corresponding to the variable (V) region, diversity and joining (DJ) region, constant (C) Ig domain, connecting peptide (CPS), transmembrane region (TM) and cytoplasmic tail (CYT) were identified in all sequences. A multiple alignment of the predicted translation of the six sea bream molecules with other known TcR $\beta$  sequences was assembled and showed the conservation of some important features from fish to mammals. Phylogenetic analysis conducted using full-length amino acid sequences evidenced that fish sequences are in a different cluster with respect to mammalian and avian ones. Real-time PCR analysis was used to investigate TcR $\beta$  basal expression, that was maximum in the thymus followed by gut, and TcR $\beta$  expression after stimulation with LPS or PHA-L at 4 and 24 h (only the 4 h stimulation with LPS gave an significant effect). Moreover, the 3D structures of TcR $\beta$  chains and class I MHC were predicted by homology modelling with the aim to investigate the amino acid residues at the interaction surface in the TcR $\beta$ /MHC complexes.

**THE T-CELL RECEPTOR BETA CHAIN FROM SEA BREAM (*Sparus aurata*): MOLECULAR CLONING, EXPRESSION AND MODELLING OF THE COMPLEXES WITH CLASS I MHC**

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## ABSTRACT

The T-cell antigen receptor (TcR) is a fundamental mediator of the adaptive immune responses, since TCR $_{\alpha\beta}$  molecules on T-cells recognize foreign structures (peptides derived from processed antigens) bound to major histocompatibility complex (MHC) antigens on another cell. In the present study we report the cDNA sequences of six TcR $\beta$  chains from sea bream (*Sparus aurata*), a fish of high economical impact in South Mediterranean aquaculture. Domains corresponding to the variable (V) region, diversity and joining (DJ) region, constant (C) Ig domain, connecting peptide (CPS), transmembrane region (TM) and cytoplasmic tail (CYT) were identified in all sequences. A multiple alignment of the predicted translation of the six sea bream molecules with other known TcR $\beta$  sequences was assembled and showed the conservation of some important features from fish to mammals. Phylogenetic analysis conducted using full-length amino acid sequences evidenced that fish sequences are in a different cluster with respect to mammalian and avian ones. Real-time PCR analysis was used to investigate TcR $\beta$  basal expression, that was maximum in the thymus followed by gut, and TcR $\beta$  expression after stimulation with LPS or PHA-L at 4 and 24 h (only the 4 h stimulation with LPS gave an significant effect). Moreover, the 3D structures of TcR $\beta$  chains and class I MHC were predicted by homology modelling with the aim to investigate the amino acid residues at the interaction surface in the TcR $\beta$ /MHC complexes.

**KEYWORDS :** T-cell antigen receptor, TcR $\beta$ , *Sparus aurata*, real time PCR, 3D structure, class I MHC, TcR $\beta$ /MHC complexes

## 1. INTRODUCTION

Cell-mediated immunity is one of the main lines of defence that vertebrates rely on for eliminating pathogen agents. T-cell receptor (TcR) antigens are present in all jawed vertebrates, while they appear to be absent in jawless fish. In mammals, TcRs are heterodimeric, consisting of either  $\alpha/\beta$  or  $\gamma/\delta$  polypeptide combination (Klausner et al., 1990). T-cells are activated when a TcR heterodimer ( $\alpha\beta$  or  $\gamma\delta$ ) in conjunction with the CD3 complex, on the surface of a T-cell, specifically recognizes an external antigen (Ag).  $\alpha\beta$  TcR recognizes Ag presented by the major histocompatibility complex (MHC) molecule expressed on antigen presenting cells (APC), while  $\gamma\delta$  TcR, that in humans is expressed only by 1-10 % of T cells, is not MHC-restricted and appears to recognize Ag in a similar manner to the direct Ag recognition processes of immunoglobulins (Ig) (Chien et al., 1996).

The TcR chains ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) are composed of variable (V), joining (J) and sometimes diversity (D) (only  $\beta$ ) segments associated in translocon type organizations with exons specifying constant domains, followed by a transmembrane domain and a short cytoplasmic tail. Recombination signal sequences and recombination activator genes (RAG) are required for rearrangement of the TcR segments (Marchalonis et al., 2002). The analysis of the TcR sequences led to the correct prediction that TcR would share a domain organization and binding mode similar to those of antibody Fab fragments (Claverie et al., 1989; Davis and Bjorkman, 1988). The first crystal structure of an  $\alpha\beta$  TcR bound to a class I MHC restricted peptide was obtained in 1996 (Fields et al., 1996) and since then on more data have been accumulated with 24 class I and class II TCR/MHC peptide complexes available at the moment (Rudolph et al., 2006). Much less is known about  $\gamma\delta$  structures and the only available data are on a quite recently obtained V $\delta$  (Li et al., 1998) domain which shows an overall architecture that closely resembles  $\alpha\beta$  TcR and Ig.

The structure and expression of genes corresponding to TcR chains have been characterised in both teleost and cartilaginous fishes (Hordvik et al., 1996; Partula et al., 1995; De Guerra and

Charlemagne, 1997; Wilson et al., 1998; Miracle et al., 2001; Wermenstam and Pilstrom, 2001; Hordvik et al., 2004; Imai et al., 2005) with the Japanese flounder (*Paralichthys olivaceus*) being the first species in which all the four genes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) have been identified (Nam et al., 2003). It has been demonstrated that TcR genes in teleost fish are organized in translocon type clusters (Zhou et al., 2003) and, in contrast to mammals, that the TcR  $\beta$  loci possess a high degree of polymorphism in the C region in bicolor damselfish (Kamper and Churchill McKinney, 2002). Moreover, the allelic polymorphism in the TcR  $\alpha$  and  $\beta$  constant domain genes is quite widespread in fishes (Criscitiello et al., 2004). Finally, the expression of TcR  $\beta$  chain has been monitored during amoebic gill disease in rainbow trout to verify its possible up/down regulation (Bridle et al., 2006).

In the present study we report the cDNA sequences of six TcR  $\beta$  chains from sea bream (*Sparus aurata*), a fish of high economical impact in South Mediterranean aquaculture, and their expression pattern both at basal level and after “in vitro” stimulations. These results will add a new tool for studying the effects of vaccinations and immuno-stimulations on the sea bream immune system. Moreover, we analysed the cDNAs organisation and predicted, by homology modelling, the 3D structures of the TcR  $\beta$  chains alone or complexed with the class I MHC.

## 2. MATERIALS AND METHODS

### 2.1 Sea bream *TcRβ* cloning and sequencing

Two degenerate primers (TCNERV: 5'- GCNGTKTAYTWCTGTGC -3' and TCNEFR: 5'- SACRTGGTCRGGRTARAA- 3' where N = A,C,G,T ; W = A,T ; R = A,G ; K = G,T ; S = G,C ; Y = C,T) corresponding to highly conserved regions of known *TcRβ* genes were used in RT-PCR on total RNA extracted with Tripure (Roche) solution from a juvenile sea bream (150 g of weight) thymus. The leukocyte cells were obtained following the procedures described in Scapigliati et al. (2001). RT-PCR was performed using Ready-To-Go RT-PCR Beads (Amersham Pharmacia). For cDNA synthesis, 1 µg of total RNA and 0.5 µg of random primers [pd(N)<sub>6</sub>] were used in each reverse transcription reaction in a total volume of 50 µl. Reactions were conducted using the Mastercycler personal (Eppendorf). The cycling protocol was one cycle of 94°C for 5 min, 35 cycles of 94°C for 45 s, 48°C for 45 s, 72°C for 45 s, followed by one cycle of 72 °C for 10 min. PCR products (15 µl) were visualised on 1% (w/v) agarose gels containing ethidium bromide (10 ng/ml) using hyperladder IV (Bioline) as size marker. Controls for the presence of DNA contamination were performed using the RNA samples as template. DNA amplified by PCR was purified using the QIAquick Gel Extraction Kit (QIAGEN), inserted into the pGEM-T Easy vector (Promega) and transfected into competent JM109 *Escherichia coli* cells. Plasmid DNA from at least ten independent clones was purified using the Wizard Plus SV Minipreps DNA Purification System (Promega) and sequenced using MWG DNA Sequencing Services. Sequences generated were analysed for similarity with other known sequences using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul et al., 1990) programs.

Further primers were designed based on the initial sea bream *TcRβ* sequence for 5'- and 3'- rapid amplification of cDNA ends (RACE)-PCR (TCRORAFR1: 5'- CCACCAACAGTGAAAGTGC -3' and TCRORAFR2: 5'-CGTTGCTTTGTGTGATCAGC-3'; TCRORARV1: 5'- GCTGATCACACAAAGCAACG -3' and TCRORARV2: 5'-GCACTTTCAGTGTGGTGG-3').



cDNA was synthesised from total thymus RNA with the First-strand cDNA Synthesis kit (Amersham Pharmacia) following the manufacturers instructions. For 3' RACE-PCR, cDNA was transcribed using an oligo-dT adaptor primer (5'-CTCGAGATCGATGCGGCCGCT<sub>15</sub>-3'). PCR was performed initially with the TCRORAFR1 primer and the oligo-dT adaptor primer, followed by a semi-nested PCR using TCRORAFR2 primer and the adaptor primer (5'-CTCGAGATCGATGCGGCCGC-3'). For 5' RACE-PCR, cDNA was transcribed from total RNA using the oligo-dT primer, treated with *E. coli* RNase H (Promega), purified using a PCR Purification Kit (QIAGEN), and tailed with poly(C) at the 5' end with terminal deoxynucleotidyl transferase (TdT, Promega). PCR was performed initially with TCRORARV1 primer and an Oligo-dG primer (5'-GGGGGGIGGGIIGGGIIG-3'), and then semi-nested with TCRORARV2 and the oligo-dG primers. Sequencing and similarity searches were as described above.

The obtained cDNA sequences were analysed for the presence of a signal peptide, using SignalP software (Nielsen et al., 1997), and of N- (with the NetNGlyc 1.0 Server) and O-linked glycosylation sites (Julenius et al., 2005). Comparison of the sea bream TcR $\beta$  amino acid sequences to their counterparts from other species was carried out using the MEGA 3.1 Software (Kumar et al., 2004). A phylogenetic tree was constructed by the “neighbour-joining” method using MEGA 3.1 Software (Kumar et al., 2004) on full-length amino acid sequences and bootstrap values calculated.

## **2.2 Basal TcR $\beta$ expression analysis**

To study the TcR $\beta$  basal expression, ten sea bream juveniles were sampled and leucocytes from different tissues (thymus, spleen, liver, gills, head kidney, gut ) obtained as described in Scapigliati et al., 2001. Total RNA was isolated from each tissue separately with Tripure (Roche) following the manufacturer's instructions, resuspended in DEPC treated water and used for real-time quantitative PCR without pooling the samples coming from the different fishes. Controls for the presence of DNA contamination were performed using  $\beta$ -actin primers that bracket an intron.

For reverse transcription, the BioScript RNase H minus (Bioline) enzyme was used with the following protocol: 2 µg of total RNA was mixed with 1 µl of random hexamer (0.2 µg/µl; Amersham Pharmacia) and nuclease free water was added to a final volume of 12 µl. This mixture was incubated at 70° C for 5 min and then cooled on ice. Successively, 0.4 µl of a reaction mix containing 100 mM dNTPs (25mM each; Promega), 4 µl of 5X Reaction buffer, nuclease free water to a final volume of 19.75 µl and 0.25 µl of BioScript at 200 u/µl were added and the solution incubated at 25 °C for 10 min and then at 37 °C for 60 min. Finally, the reaction was stopped by heating at 70 °C for 10 min.

The expression level of TcRβ was determined with a Mx3000P™ real time PCR system (Stratagene) equipped with version 2.02 software and using the Brilliant SYBR Green Q-PCR Master Mix (Stratagene) following the manufacturer's instructions, with ROX as internal passive reference dye. The reference dye is not reactive during real time PCR and therefore can be used to normalize slight differences in the volume of the added real time PCR reaction, transparency of the plastic caps and other sources of well-to-well differences. Specific PCR primers were designed for the amplification of about 200 bp products from both TcRβ (selected in the Ig constant region) and β-actin, used as an house-keeping gene. The primers were: RQTCRORAFW2: 5'-GTCGACGCCAACAATGG- 3' AND RQTCRORARW2: 5'-AGACGCAGCCCTTGATGA-3', RTACTFR2: 5'-ATGTACGTTGCCATCC-3' and RTACTRV2: 5'-GAGATGCCACGCTCTC-3', respectively. Approximately 20 ng of cDNA template was used in each PCR reaction. The PCR conditions were 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s. Triplicate reactions were performed for each template cDNA and the template was replaced with water in all blank control reactions. Each run was terminated with a melting curve analysis which resulted in a melting peak profile specific for the amplified target DNA. The analysis was carried out using the endpoints method option of the Mx3000P™ software that causes the collection of the fluorescence data at the end of each extension stage of amplification. A relative

quantitation has been performed, comparing the levels of the target transcript (TcR $\beta$ ) to a reference transcript (calibrator, the tissue with the lowest TcR $\beta$  expression, in this case the head kidney) with an expression level of the gene of interest (TcR $\beta$ ) defined as 1.0. A normalizer target ( $\beta$ -actin) is included to correct for differences in total cDNA input between samples. The quantitative experiment is based on threshold cycle (Ct) determination, defined as the cycle at which a statistically-significant increase in fluorescence (above background signal) is detected. The results are expressed as the mean  $\pm$  SD of the results obtained from the ten considered fishes. The real-time PCR products from the different tissues were examined successively by agarose gel electrophoresis to investigate their specificity and size.

### **2.3 *In vitro* TcR $\beta$ expression analysis**

The *in vitro* TcR $\beta$  expression was studied by real time PCR using different stimulating conditions on head kidney (HK) leucocytes obtained from ten sea bream juveniles as described above. HK leucocytes were adjusted to  $1 \times 10^5$  cells/ml and incubated at 18 °C for 4 h and 24 h with 5  $\mu$ g/ml of lipopolysaccharide (LPS from *E. coli* 0127:B8, Sigma) in PBS or with 1  $\mu$ g/ml of lectin from *Phaseolus vulgaris* Leucoagglutinin (PHA-L from Sigma) in PBS. The control was stimulated with PBS only at the same time points.

Total RNA was isolated with Tripure (Roche) following the manufacturer's instructions, resuspended in DEPC treated water and used for real-time quantitative PCR without pooling the samples coming from the different fishes. The primers and the real time PCR conditions were the same as described above, except that the calibrators for these experiments were the time 0 control. Data were expressed as the mean  $\pm$  SD of the results obtained from the ten considered fishes and the differences from the control have been considered significant if  $p < 0.05$  using the standard *t* student test.

### **2.4 3D Modelling of sea bream TcR $\beta$ chains**

The 3D models of the six sea bream TcR $\beta$  sequences (region 21-140) were created according to the homology modelling strategy and using as templates the experimental human and murine structures of TcR $\beta$  complexed with HLA-A2 (PDB code: 2GJ6, chain E) (Gagnon et al., 2006) and I-Ak proteins (PDB code: 1G6R, chain B) (Degano et al., 2000), respectively. As the sequence identities between the sea bream TcR $\beta$  chains and the human and murine homologous templates were close to 30%, we used an accurate procedure for the modelling strategy (Facchiano et al., 2001; Scapigliati et al., 2004; Costantini et al., 2005; Buonocore et al., 2006; Buonocore et al., 2007; Costantini et al., 2007). The search for sequence similarity within databases was performed with the BLAST program (Altschul et al., 1990). The alignment of the protein sequences was made with CLUSTALW program (Thompson et al., 1994) and a few manual refinements were added to account for the position of secondary structures.

The MODELLER module (Sali and Blundell, 1993) implemented in the Quanta molecular simulation package (Accelrys Inc., San Diego, CA, USA) was used to build 10 full-atom models of each TCR $\beta$  chain by setting 4.0 Angstroms as RMS deviation among initial models and by full optimization of models. To select the best model, we used the PROCHECK program (Laskowski et al., 1993) to evaluate their stereochemical quality and structural packing quality, and ProsaII program (Sippl, 1993) to check the fitness of the sequences to the obtained structures and to assign a scoring function. Secondary structures were assigned by the DSSP program (Kabsch and Sander, 1983). Search for structural classification was performed on CATH database (Orengo et al., 1997; Pearl et al., 2000). Molecular superimposition, RMSD values and figures were obtained with the Insight II package (Accelrys Inc., San Diego, CA, USA).

### ***2.5 3D Modelling of sea bream class I MHC***

The modelling of sea bream MHC class I (20-294 amino acid region, accession number DQ211541) was performed using as template the experimental human and murine structures of MHC class I complexed with TcR $\beta$  (PDB code: 2GJ6, chain A, and 1G6R, chain H) (Gagnon et al.,

2006; Degano et al., 2000). The 3D structure of sea bream MHC class I was modelled and analysed with the same procedures and software used for TcR $\beta$  chains.

## ***2.6 Simulation of sea bream class I MHC/TcR $\beta$ complexes***

The sea bream MHC class I/TcR $\beta$  complexes were created using as reference the experimental models of human and murine MHC class I complexed with the related TcR $\beta$  chains (Gagnon et al., 2006; Degano et al., 2000).

CVFF force field developed for the Insight II software was adopted to assign potentials and charges for the complexed proteins. The complexes were minimized by using 500 steps of energy minimization under conjugate gradient algorithm in order to optimise side chain conformations and avoid sterical clashes (Scapigliati et al., 2004; Costantini et al., 2005; Chambery et al., 2007; Costantini et al., 2007; Gianfrani et al., 2007). To compare the protein-protein interaction in the complexes, the “Protein - Protein Interaction Server” (Jones and Thornton, 1996) and the program NACCESS (Hubbard et al., 1991) were used to evaluate the interface surface area and to identify the amino acids at the protein-receptor interface. H-bonds were calculated with Hbplus program (McDonald and Thornton, 1994), that identifies H-bonds within a distance of 2.5 Å and a minimum angle of 90°. Moreover, the energy of interaction between MHC and TcR $\beta$  was also calculated by using the Energy/Intermolecular tool in the Docking module of Insight II and the binding free energy by using the DCOMPLEX program (Liu et al., 2004).

### 3. RESULTS

#### 3.1 Sea bream TcR $\beta$ sequences analysis

PCR with degenerate primers TCNEFR and TCNERV gave a product of the expected size (219 bp) that when sequenced showed high homology for other known TcR $\beta$  sequences (data not shown). 3'-RACE-PCR performed with TCRORAFR2 (based on the initial 219 bp sequence) and the adaptor primer gave a product of about 927 bp that contained the 3'-end of the gene. 5'-RACE-PCR performed with TCRORARV2 (based on the initial 219 bp sequence) and oligo- dG gave a product of about 476 bp that contained the 5'-end of the gene, with some differences between the selected clones. The six full-length nucleotide sequences (EMBL accession numbers AM261209; AM261210; AM490435; AM490436; AM490437; AM490438) are comprised of 1428 bp from the three overlapping products and have been confirmed by PCR using primers that amplify the coding sequence (data not shown). They encode for 308 amino acids, with a predicted 21 amino acid signal peptide, a 44 bp 5'-UTR and a 482 bp 3'-UTR. Finally, the 3'-UTR contained a polyadenylation signal (AATAAA) 18 bp upstream of the poly(A) tail.

A multiple alignment of the predicted translation of the six sea bream molecules with other known TcR $\beta$  sequences was assembled (Fig. 1) to investigate the conservation of characteristic amino acid residues involved in structural domains found within TcRs. Domains corresponding to the variable (V) region, diversity and joining (DJ) region, constant (C) Ig domain, connecting peptide (CPS), transmembrane region (TM) and cytoplasmic tail (CYT) were identified according to Hein (1994). An analysis of the *Sparus aurata* clone 1 sequence (Fig. 1) showed the presence of four putative N-glycosylation sites, one in the DJ region and three in the C domain, and no O-glycosylation sites. A similar situation for the putative glycosylation sites has been found in the channel catfish (*Ictalurus punctatus*) (Wilson et al., 1997), in the Atlantic salmon (*Salmo salar*) (Hordvik et al., 1996) and in the Japanese flounder (*Paralichthys olivaceus*) (Nam et al., 2003).

Some amino acid residues are conserved in all the considered sequences (Fig. 1) from fish to mammals and this should indicate their importance for the functional activity of TcR $\beta$  molecules.

The two cysteine residues that in human (C-42/C-110) are involved in an intrachain disulfide bond fundamental for the assembly of the V domain (Wilson and Garcia, 1997) are conserved in sea bream, together with the sequence Phe<sup>122</sup>-Gly<sup>123</sup>-X<sup>124</sup>-Gly<sup>125</sup> in the DJ region, typical of most TcRs and Ig light chains (Wilson et al., 1998). The constant Ig domain is the region that presents the highest homology between all considered sequences. The two cysteine residues involved in human (C-162/C-227) in the formation of another intrachain disulfide bond (Wilson and Garcia, 1997), the sequence Ser<sup>211</sup>-Arg<sup>212</sup>-Leu<sup>213</sup>, linked to the TcR $\alpha/\beta$  interaction (Arnaud et al., 1997), and a Glu residue (E-151 in human), which is thought to form an hydrophilic bond with the TcR $\alpha$  chain (Garcia et al., 1996), are conserved in sea bream. On the contrary, the Cys residue that in mammals (Wilson and Garcia, 1997) is known to form an interchain disulphide bond with TcR $\alpha$  chain is not present, as already observed in all known teleost sequences (Hordvik et al., 1996; Wilson et al., 1998; Wermenstam and Pilstrom, 2001; Nam et al., 2003). The CPS shows no conserved amino acids, while in the TM region the residue K<sup>280</sup>, that should be important for the TcR cell surface expression and for the assembly of the complex between TcR $\beta$  and CD3 (Morley et al., 1988; Alcover et al., 1990), is present in sea bream.

Phylogenetic analysis (Fig. 2) conducted using full-length amino acid sequences showed that fish sequences are in a different cluster with respect to mammalian and avian sequences. The six sea bream TcR $\beta$  sequences are all in the same cluster but divided in four different groups: *Sparus aurata* clone 2 and clone 3 (with 93 % sequence identity), and *Sparus aurata* clone 1 and clone 6 (with 94 % sequence identity) are in the same group, whereas *Sparus aurata* clone 4 and *Sparus aurata* clone 5 are in two different groups.

### **3.2 Basal TcR $\beta$ expression analysis**

The expression analysis of TcR $\beta$  in organs and tissues of un-stimulated sea bream is shown in Figure 3. Real-time PCR products were loaded on agarose gels to exclude the formation of non-specific amplicons and single bands of the expected sizes were obtained. Moreover, to take into

consideration the individual genetic variability ten different fish were sampled and analysed separately. The highest TcR $\beta$  expression was detected in thymus, followed by gut. Lower TcR $\beta$  mRNA levels were observed in spleen, liver and gills. The head kidney showed the lowest expression level.

### **3.3 *In vitro* TcR $\beta$ expression analysis**

To investigate whether TcR $\beta$  expression level could be modulated with LPS and PHA-L, *in vitro* stimulation of HK leucocytes for a short (4 h) and a longer (24 h) time was studied; RNA was extracted from the HK leucocytes of ten individuals analysed separately. Real-time PCR products were loaded on agarose gels and single bands of the expected sizes were obtained. The results are shown in Figure 4. Using LPS (Fig. 4), a dramatic increase of TcR $\beta$  expression was detected at 4 h ( $p < 0,05$ ), whereas at 24 h ( $p < 0,05$ ) it was similar to the control. When using PHA for *in vitro* treatment (Fig. 4), a little decrease was observed both at 4 h and 24 h, although being not statistically significant.

### **3.4 3D Modelling of sea bream TcR $\beta$ chains**

The crystallographic structures of human and mouse TcR $\beta$  chains, complexed with HLA-A2 (PDB code: 2GJ6, chain E) (Gagnon et al., 2006) and I-Ak (PDB code: 1G6R, chain B) (Degano et al., 2000) respectively, were selected as template models as the sequence identity percentages between sea bream and man/mouse TcR $\beta$  molecules ranged from 28% to 35%. The alignments of the sea bream TcR $\beta$  sequences with human and murine ones and the related experimental structures (data not shown) were used to create structural models for each sea bream TcR $\beta$  chain for the region between the amino acid 21 and 140. In Figure 5 the 3D structure for the TcR $\beta$  *Sparus aurata* clone 3 (the sequence with the highest similarity with human and murine templates) with its secondary structure elements is shown. About 50% of the residues in each sea bream TcR $\beta$  model folds in beta conformation, thus defining the global structure as “mainly beta” with immunoglobulin-like topology in agreement to the structural classification reported by CATH



database (Orengo et al., 1997) for the reference structures of human and murine TCR $\beta$ . In each TCR $\beta$  molecule the position and orientation of two Cys residues make it possible that the structure is stabilized by an intrachain disulfide bond. The short  $3_{10}$  helix observed in both crystallographic human and murine structures is present also in all sea bream models.

The TCR $\beta$  models were compared by superimposition of structurally conserved regions, obtaining root mean square deviation (RMSD) values related to the differences in terms of secondary structures. RMSD values range between 0.42 and 0.99 Angstroms, indicating that these conformations present some structural differences, even if the overall 3D structure is conserved.

### ***3.5 3D Modelling of sea bream class I MHC***

The search in the non-redundant database evidenced that the 3D structure of HLA-A2 (PDB code: 2GJ6, chain A) (Gagnon et al., 2006) and I-Ak (PDB code: 1G6R, chain H) (Degano et al., 2000) can be used to obtain the 3D model of sea bream class I MHC as the sequence identity percentages between sea bream and man/mouse resulted of 31 and 34%, respectively. Starting from the alignment of these three sequences (data not shown), ten structural models were created for sea bream class I MHC 20-294 amino acid region and the best model (Figure 6) was selected as described before (see the section: “Materials and Methods). This model has a classical organization in three distinct domains (i.e.  $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$ ) in agreement with the structural classification reported by CATH database (Orengo et al., 1997; Pearl et al., 2000) for the crystallographic structures of human and murine class I MHC molecules. The  $\alpha 1$  and  $\alpha 2$  domains are classified as “alpha-beta” and each consists of an alpha helical region and four strands of beta sheet in an anti-parallel orientation. The C-terminal domain ( $\alpha 3$ ) has a “mainly beta” fold and is characterized by an immunoglobulin-like beta-sandwich made of two anti-parallel sheets, each consisting of three main strands and few shorter strands, organized in greek-keys motifs. In the sea bream class I MHC model four cysteine residues are located in the same positions of the human and murine templates and they should form two intrachain disulfide bonds, one in the  $\alpha 2$  domain and the other in the  $\alpha 3$

domain. The sea bream class I MHC model was compared by structural superimposition, as for TCR $\beta$  models, with the two experimental structures used as templates, but the presence of gaps in the alignment made it difficult to perform a complete structural comparison of the three models (RMSD values of 1.12 and 0.96, respectively). However, the comparison of the secondary structures evidenced that the helices and  $\beta$ -strands are conserved, with few external residues resulting added or excluded in some secondary structure elements and that the  $3_{10}$  helix present in human and murine structures is not conserved in the sea bream class I MHC model.

### ***3.6 Simulation of sea bream class I MHC/TcR $\beta$ complexes***

On the basis of the crystallographic structures of the human and murine class I MHC/TcR $\beta$  complexes we simulated the interaction between the six TcR $\beta$  chains and class I MHC in sea bream according to the procedure already described (Scapigliati et al., 2004; Costantini et al., 2005; Chambery et al., 2007; Costantini et al., 2007; Gianfrani et al., 2007). For each complex we evaluated the interaction residues, the number of intrachain H-bonds and the interface surface area (Table I). The interaction regions between the class I MHC and TCR $\beta$  in each complex are well conserved (data not shown) and, in particular, the amino acids of TcR $\beta$  chains at the interface with class I MHC are located in loop regions, whether the class I MHC ones in helical regions. The number of interaction residues is different in the several complexes, due to the amino acid differences between the six sea bream TcR $\beta$  amino acid sequences. The value of interface surface area and the number of intrachain H-bonds resulted the highest for the complex between class I MHC and TcR $\beta$  clone 3 (see Table I); the other complexes show little differences in the interface surface area values and variable numbers of intrachain H-bonds.

#### 4. DISCUSSION

The heterodimeric, membrane anchored TcR endows the cell with antigen specific reactivity and this molecule has been extensively studied in mammals. Fish, like all jawed vertebrate, can develop cell mediated immunity and the knowledge on fish TcR has increased rapidly in recent years.

In this paper we report the homology cloning of TcR $\beta$  chain from sea bream (*Sparus aurata*), and analysed its expression and 3D structure, with a particular interest to the interactions with class I MHC.

Six different TcR $\beta$  cDNAs were isolated from the thymus of a juvenile sea bream and the predicted amino acid sequences were studied to evidence the presence of conserved structures. The J segment motif (Moss et al., 1995), F-G-X-G, and the four Cys residues involved in mammals in two intrachain disulfide bonds are conserved in all sea bream sequences (see Figure 1). The Cys that in mammals is involved in an interchain disulfide bond with TcR $\alpha$  is not present in sea bream, but some authors (Arnaud et al., 1997) demonstrated that it may be not important for the dimerisation of the TcR $\alpha\beta$  heterodimer. On the contrary, the amino acid motif (YCLSSRLRVSA) that the same authors proposed to be involved in the  $\alpha\beta$  chain interaction and cell surface expression, is partially conserved in all sequences. Therefore, the amino acids (the Y and the stretch SRL) that in this motif are retained from fish to mammals should be fundamental for the structure/function relationship in TcR $\beta$ s. The conserved antigen receptor transmembrane (CART) motif (Campbell et al., 1994), made mainly of polar or aromatic amino acids, is present in sea bream TM sequences like in all teleost TcR $\beta$ . It should encode a structural unit with an important role in the assembly and/or signalling of the TcR/CD3 complex (Campbell et al., 1994). In particular, the two tyrosine residues (Y-265 and Y-275) that have been identified to have a fundamental role in the signalling of TcR $\beta$  towards the  $\xi$  subunit of CD3 (Fuller-Espie et al., 1998) are conserved in sea bream.

Phylogenetic analysis (see Fig. 2) evidenced that, although some features are conserved, fish are in a different cluster compared to mammals and sea bream TcR $\beta$  sequences are inside this cluster, with bicolor damselfish and rainbow trout as the most closely related.

Real-time PCR analysis detected high levels of constitutive TcR $\beta$  expression in thymus (see Fig. 3), as already observed in cod (Wermenstam and Pilstrom, 2001) and rainbow trout (Partula et al., 1995), followed by gut, in contrast to what was detected in cod (Wermenstam and Pilstrom, 2001) where no expression was found in the intestine. However, the gut is a tissue with a certain number of T-cells (Romano et al., 2007) and deeply involved in fish immune responses and leukocytes ontogeny as demonstrated in rainbow trout (Bernard et al., 2006), sea bass and carp (Rombout et al., 2005). The *in vitro* TcR $\beta$  expression was studied using sea bream head kidney leukocyte cells and the selected stimulants were LPS, to simulate a pathogen infection, and the cell mitogen agent PHA-L. LPS induced an high TcR $\beta$  expression in sea bream after 4 h as observed also *in vivo* on mice (Tough et al., 1997), whether PHA-L showed no effect after both at 4 h and 24 h. Probably, this last result was due to the stimulation period that was too short, as in the *Pagrus auratus* (Morrison et al., 2004) leukocyte proliferation was induced significantly by PHA only after 72 h.

3D structural analysis of sea bream TcR $\beta$  chains showed that the beta-strands are well conserved in all models, although some little differences could be evidenced. In fact, few external residues result added or excluded in the  $\beta$ -strands, and, in TCR $\beta$  clone 1 and clone 2 a further short  $\beta$ -strand in the 57-58 residue region is un-recognized (data not shown). The simulation of the complexes between the TcR $\beta$  chains and the class I sea bream MHC gave us the opportunity to study the amino acid residues at the interaction surface. Furthermore, for each complex we have evaluated the binding energy and interaction energy (Fig. 7) and the best values have been obtained for the complex with TcR $\beta$  clone 3: this complex showed also the highest value of interface surface area and the highest number of intrachain H-bonds (see Table I).

In conclusion, the availability of sea bream TcR $\beta$  sequences will add a new tool to analyse fish immune responses to viral and parasitic infections and will be of relevance to study the phylogeny and evolution of specific antigen receptors. Moreover, the investigation on the TcR $\beta$ /class I MHC complexes will help to have information about the putative amino acid residues at the interaction surfaces. The step forward will be the search of an experimental testing to assess the 3D structures reliability: probably it could be possible to make some synthetic peptides that should block the interactions between TcR $\beta$  and class I MHC, based on the conserved amino acid residues at the interaction surfaces, and investigate if these peptides are able to modulate sea bream immune responses.

#### **ACKNOWLEDGEMENTS**

Authors are indebted to Dr. C. Magugliani and Dr. E. Vela (Nuova Azzurro, Civitavecchia) for the supply of fish. This work was supported by the European Commission within the project IMAQUANIM (EC contract number FOOD-CT-2005-007103).

## REFERENCES

- Alcover, A., Mariuzza, R.A., Ermonval, M., Acuto, O., 1990. Lysine 271 in the transmembrane domain of the T-cell antigen receptor  $\beta$  chain is necessary for its assembly with the CD3 complex but not for  $\alpha/\beta$  dimerization. *J. Biol. Chem.* 265, 4131-4135.
- Altschul, S.F., Gish, W., Miller, W., Myers, E., Lipman, D.J., 1990. Best local alignment search tool. *J. Mol. Biol.* 215, 403-410.
- Arnaud, J., Hucheq, A., Vernhes, M.-C., Caspar-Bauguil, S., Lenfant, F., Sancho, J., Terhost, C., Rubin, B., 1997. The interchain disulfide bond between TCR $\alpha\beta$  heterodimers on human T cells is not required for TCR-CD3 membrane expression and signal transduction. *Inter. Immunol.* 9, 615-626.
- Bernard, D., Six, A., Rigottier-Gois, L., Messiaen, S., Chilmonczyk, S., Quillet, E., Boudinot, P., Benmansour, A., 2006. Phenotypic and functional similarity of gut intraepithelial and systemic T cells in a teleost fish. *J. Immunol.* 176, 3942-3949.
- Bridle, A.R., Morrison, R.N., Nowak, B.F., 2006. The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss*, during amoebic gill disease (AGD). *Fish Shellfish Immunol.* 20, 346-364.
- Buonocore, F., Randelli, E., Bird, S., Secombes, C.J., Costantini, S., Facchiano, A., Mazzini, M., Scapigliati, G., 2006. The CD8 $\alpha$  from sea bass (*Dicentrarchus labrax* L.): cloning, expression and 3D modelling. *Fish Shellfish Immunol.* 20, 637-646.
- Buonocore, F., Randelli, E., Casani, D., Costantini, S., Facchiano, A., Scapigliati, G., Stet, R.J., 2007. Molecular cloning, differential expression and 3D structural analysis of the MHC class-II beta chain from sea bass (*Dicentrarchus labrax* L.). *Fish Shellfish Immunol.*, in press.
- Campbell, K.S., Backstrom, B.T., Tiefenthaler, G., Palmer, E., 1994. CART: a conserved antigen receptor transmembrane motif. *Semin. Immunol.* 6, 393-410.

503 Chambery, A., Pisante, M., Di Maro, A., Di Zazzo, E., Costantini, S., Colonna, G., Parente, A.,  
 504 2007. Invariant Ser211 is involved in the catalysis of PD-L4, type I RIP from (*Phytolacca dioica*)  
 505 leaves. *Proteins: Structure, Function and Bioinformatics* 67, 209-218.

506 Chien, Y.H., Jores, R., Crowley, M.P., 1996. Recognition by  $\gamma/\delta$  T cells. *Annu. Rev. Immunol.*  
 507 14, 511-532.

508 Claverie, J.M., Prochnicka-Chalufour, A., Bougueleret, L., 1989. Implications of a Fab-like  
 509 structure for the T-cell receptor. *Immunol. Today* 10, 10-14.

510 Costantini, S., Colonna, G., Facchiano, A.M., 2007. Simulation of conformational changes  
 511 occurring when a protein interacts with its receptor. *Comput. Biol. Chem.* 31, 196-206.

512 Costantini, S., Colonna, G., Rossi, M., Facchiano, A.M., 2005. Modelling of HLA-DQ2 and  
 513 simulations of its interaction with gluten peptides to explain molecular recognition in celiac disease.  
 514 *J. Mol. Graph. Model.* 23, 419-431.

515 Criscitiello, M.F., Wermenstam, N.E., Pilstrom, L., McKinney, E.C., 2004. Allelic  
 516 polymorphism of T-cell receptor constant domains is widespread in fishes. *Immunogenetics* 55,  
 517 818-824.

518 Davis, M.M., Bjorkmann, P.J., 1988. T-cell antigen receptor genes and T-cell recognition.  
 519 *Nature* 334, 395-402.

520 Degano, M., Garcia, K.C., Apostolopoulos, V., Rudolph, M.G., Teyton, L., Wilson, I.A., 2000.  
 521 A functional hot spot for antigen recognition in a superagonist TCR/MHC complex. *Immunity* 12,  
 522 251-261.

523 De Guerra, A., Charlemagne, J., 1997. Genomic organisation of the TcR  $\beta$ -chain diversity ( $D\beta$ )  
 524 and joining ( $J\beta$ ) segments in the rainbow trout: presence of many repeated sequences. *Mol.*  
 525 *Immunol.* 34, 653-662.

526 Facchiano, A.M., Stiuso, P., Chiusano, M.L., Caraglia, M., Giuberti, G., Marra, M., Abruzzese,  
 527 A., Colonna, G. 2001. Homology modelling of the human eukaryotic initiation factor 5A (eIF-5A).  
 528 *Protein Engineering* 14, 881-890.

529 Fields, B.A., Malchiodi, E.L., Li, H., Ysern, X., Stauffacher, C.V., Schlievert, P.M.,  
530 Karjalainen, K., Mariuzza, R.A., 1996. Crystal structure of a T-cell receptor  $\beta$ -chain complexed  
531 with a superantigen. *Nature* 384, 188-192.

532 Fuller-Espie, S., Hoffman Towler, P., Wiest, D.L., Tietjen, I., Spain, L.M., 1998.  
533 Transmembrane polar residue of TCR  $\beta$  chain are required for signal transduction. *Inter. Immunol.*  
534 10, 923-933.

535 Gagnon, S.J., Borbulevich, O.Y., Davis-Harrison, R.L., Turner, R.V., Damirjian, M.,  
536 Wojnarowicz, A., Biddison, W.E., Baker, B.M., 2006. T cell receptor recognition via cooperative  
537 conformational plasticity. *J. Mol. Biol.* 363, 228-243.

538 Garcia, K.C., Degano, M., Stanfield, R.L., Brunmark, A., Jackson, M.R., Peterson, P.A.,  
539 Teyton, L., Wilson, I.A., 1996. An  $\alpha\beta$  T cell receptor structure at 25 Å and its orientation in the  
540 TCR-MHC complex. *Science* 274, 209-219.

541 Gianfrani, C., Siciliano, R., Facchiano, A.M., Camarca, A., Mazzeo, F.M., Costantini, S.,  
542 Savati, V.M., Maurano, F., Mazzeo, G., Iaquinto, G., Bergamo, P., Rossi, M., 2007.  
543 Transamidation of wheat flour inhibits the response to gliadin of intestinal T cells in celiac disease.  
544 *Gastroenterology*, in press.

545 Hein, W.R., 1994. Structural and functional evolution of the extracellular regions of T cell  
546 receptors. *Semin. Immunol.* 6, 361-372.

547 Hordvik, I., Jacob, A.L.J., Charlemagne, J., Endresen, C., 1996. Cloning of T-cell antigen  
548 receptor beta chain cDNAs from Atlantic salmon (*Salmo salar*). *Immunogenetics* 45, 9-14.

549 Hordvik, I., Torvund, J., Moore, L., Endresen, C., 2004. Structure and organisation of the T cell  
550 receptor alpha chain genes in Atlantic salmon. *Mol. Immunol.* 41, 553-559.

551 Hubbard, S.J., Campbell, S.F., Thornton, J.M., 1991. Molecular recognition. Conformational  
552 analysis of limited proteolytic sites and serine proteinase protein inhibitors. *J. Mol. Biol.* 220, 507-  
553 530.



554 Imai, E., Ishikawa, J., Moritomo, T., Tomana, M., 2005. Characterisation of T cell antigen  
555 receptor  $\alpha$  chain isotypes in the common carp. *Fish Shellfish Immunol.* 19, 205-216.

556 Jones, S., Thornton, J.M., 1996. Principles of protein-protein interactions derived from  
557 structural studies. *Proc. Natl. Acad. Sci. USA* 93, 13-20.

558 Julenius, K., Molgaard, A., Gupta, R., Brunak, S., 2005. Prediction, conservation analysis and  
559 structural characterization of mammalian mucin-type O-glycosylation sites. *Glycobiology* 15, 153-  
560 164.

561 Kabsch, W., Sander, C., 1983. Dictionary of protein secondary structure: pattern recognition of  
562 hydrogen-bonded and geometrical features. *Biopolymers* 22, 2577-2637.

563 Kamper, S.M., Churchill McKinney, E.C., 2002. Polymorphism and evolution in the constant  
564 region of the T-cell receptor beta chain in an advanced teleost fish. *Immunogenetics* 53, 1047-1054.

565 Klausner, R.D., Lippincott-Schwartz, J., Bonifacino, J.S., 1990. The T-cell antigen receptor:  
566 insights into organelle biology. *Annu. Rev. Cell Biol.* 6, 403-431.

567 Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated Software for Molecular  
568 Evolutionary Genetics Analysis and Sequence Alignment. *Briefings in Bioinformatics* 5, 150-163.

569 Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK - A  
570 program to check the stereochemical quality of protein structures. *J. Appl. Cryst.* 26, 283-291.

571 Li, H., Lebedeva, M.I., Llera, A.S., Fields, B.A., Brenner, M.B., Mariuzza, R.A., 1998.  
572 Structure of the V $\delta$  domain of a human  $\gamma\delta$  T-cell antigen receptor. *Nature* 291, 502-506.

573 Liu, S., Zhang, C., Zhou, H., Zhou, Y., 2004. A physical reference state unifies the structure-  
574 derived potential of mean force for protein folding and binding. *Proteins* 56, 93-101.

575 Marchalonis, J.J., Jensen I., Schulter S.F., 2002. Structural, antigenic and evolutionary analyses  
576 of immunoglobulins and T cell receptors. *J. Mol. Recognit.* 15, 260-271.

577 McDonald, I.K., Thornton, J.M., 1994. Satisfying hydrogen bonding potential in proteins. *J.*  
578 *Mol. Biol.* 238, 777-793.

579 Miracle, A.L., Anderson, M.K., Litman, R.T., Walsh, C.J., Luer, C.A., Rothenberg, E.V.,  
580 Litman, G.W., 2001. Complex expression patterns of lymphocyte-specific genes during the  
581 development of cartilaginous fish implicate unique lymphoid tissues in generating an immune  
582 repertoire. Intern. Immunol. 13, 567-580.

583 Morley, B.J., Chin, K.N., Newton, M.E., Weiss, A., 1988. The lysine residue in the membrane-  
584 spanning domain of the  $\beta$  chain is necessary for cell surface expression of the T-cell antigen  
585 receptor. J. Exp. Med. 168, 1971-1978.

586 Morrison, R.N., Lyons, A.B., Nowak, B.F., Hayball, J.D., 2004. Snapper (*Pagrus auratus*)  
587 leucocyte proliferation is synergistically enhanced by simultaneous stimulation with LPS and PHA.  
588 Fish Shellfish Immunol. 16, 307-319.

589 Moss, P.A., Bell, J.I., 1995. Sequence analysis of the human  $\alpha\beta$  T-cell receptor CDR3 region.  
590 Immunogenetics 42, 10-18.

591 Nam, B-H., Hirono, I., Aoki, T., 2003. The four TCR genes of teleost fish: the cDNA and  
592 genomic DNA analysis of Japanese flounder (*Paralichthys olivaceus*) TCR  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -chains.  
593 J. Immunol. 170, 3081-3090.

594 Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and  
595 eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1-6.

596 Orengo, C.A., Michie, A.D., Jones, S., Jones, D.T., Swindells, M.B., Thornton, J.M., 1997.  
597 CATH- A Hierarchic Classification of Protein Domain Structures. Structure 5, 1093-1108.

598 Partula, S., De Guerra, A., Fellah, J.S., Charlemagne, J., 1995. Structure and diversity of the T  
599 cell antigen receptor  $\beta$ -chain in a teleost fish. J. Immunol. 155, 699-706.

600 Pearl, F.M.G., Lee, D., Bray, J.E., Sillitoe, I., Todd, A.E., Harrison, A.P., Thornton, J.M.,  
601 Orengo, C.A., 2000. Assigning genomic sequences to CATH. Nucl. Acids Res. 28, 277-282.

602 Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. Proc.  
603 Natl. Acad. Sci. USA 85, 2444-2448.

604 Romano, N., Rossi, F., Abelli, L., Caccia, E., Piergentili, R., Mastrolia, L., Randelli, E.,  
605 Buonocore, F., 2007. Majority of TcR $\beta$ (+) T-lymphocytes located in the thymus and midgut of the  
606 bony fish, *Dicentrarchus labrax* (L.). Cell Tissue Res., in press.

607 Rombout, J.H., Huttenhuis, H.B., Picchietti, S., Scapigliati, G., 2005. Phylogeny and ontogeny  
608 of fish leucocytes. Fish Shellfish Immunol. 19, 441-455.

609 Rudolph, M.G., Stanfield, R.L., Wilson, I.A., 2006. How TCRs Bind MHCs, peptides and  
610 coreceptors. Annu. Rev. Immunol. 24, 419-466.

611 Sali, A., Blundell, T.L., 1993. Comparative protein modelling by satisfaction of spatial  
612 restraints. J. Mol. Biol. 234, 779-815.

613 Scapigliati, G., Buonocore, F., Bird, S., Zou, J., Pelegrin, P., Falasca, C., Prugnoli, D.,  
614 Secombes, C.J., 2001. Phylogeny of cytokines: molecular cloning and expression analysis of sea  
615 bass *Dicentrarchus labrax* interleukin-1 beta. Fish Shellfish Immunol. 11, 711-726.

616 Scapigliati, G., Costantini, S., Colonna, G., Facchiano, A., Buonocore, F., Bossù, P., Holland,  
617 J.W., Secombes, C.J., 2004. Modelling of fish interleukin 1 and its receptor. Dev. Comp. Immunol.  
618 28, 429-441.

619 Sippl, M.J., 1993. Recognition of errors in three-dimensional structures of proteins. Proteins 17,  
620 355-362.

621 Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: Improving the sensitivity of  
622 progressive multiple sequence alignment through sequence weighting, position-specific gap  
623 penalties and weight matrix choice. Nucl. Acids Res. 22, 4673-4680.

624 Tough, D.F., Sun, S., Sprent, J., 1997. T cell stimulation *in vivo* by lipopolysaccharide (LPS). J.  
625 Exp. Med. 185, 2089-2094.

626 Wermenstam, N.E., Pilstrom, L., 2001. T-cell antigen receptors in Atlantic cod (*Gadus morhua*  
627 L.): structure, organisation and expression of TCR  $\alpha$  and  $\beta$  genes. Dev. Comp. Immunol. 25, 117-  
628 135.

Wilson, I.A., Garcia, K.C., 1997. T-cell receptor structure and TCR complexes. Curr. Opin. Struct. Biol. 7, 839-848.

Wilson, M.R., Zhou, H., Bengten, E., Clem, L.W., Stuge, T.B., Warr, G.W., Miller, N.W., 1998. T-cell receptors in channel catfish: structure and expression of TCR  $\alpha$  and  $\beta$  genes. Mol. Immunol. 35, 545-557.

Zhou, H., Bengten, E., Miller, N.W., Clem, L.W., Wilson, M., 2003. The T cell receptor  $\beta$  locus of the channel catfish, *Ictalurus punctatus*, reveals unique features. J. Immunol. 170, 2573-2581.

## FIGURE CAPTIONS

**Figure 1.** Alignment of the predicted sea bream TcR $\beta$  amino acid sequences with other known TcR $\beta$  molecules. Regions corresponding to the putative signal peptide, variable region, diversity and joining region, constant Ig domain, connecting peptide (CPS), transmembrane region (TM) and cytoplasmic tail (CYT) are shown above the sequences. Conserved cysteine residues are evidenced in bold, conserved amino acid residues are indicated with an asterisk, the putative N-glycosylation sites are in bold and underlined. Accession numbers: *Sparus aurata* (sea bream) clone 1 AM261209; *Sparus aurata* clone 2 AM261210; *Sparus aurata* clone 3 AM490435; *Sparus aurata* clone 4 AM490436; *Sparus aurata* clone 5 AM490437; *Sparus aurata* clone 6 AM490438; *Gadus morhua* (Atlantic cod) AJ133848; *Stegastes partitus* (bicolor damselfish) AAG46047; *Paralichthys olivaceus* (bastard halibut) AB053414; *Oncorhynchus mykiss* (rainbow trout) AF329700; *Anas platyrhynchos* (mallard) AY039002; *Rattus norvegicus* (Norway rat) BC099166; *Mus musculus* (house mouse) AY188690; *Homo sapiens* (human) DQ341458.

**Figure 2.** Phylogenetic tree showing the relationship between the six sea bream TcR $\beta$  sequences and other known TcR $\beta$  molecules. The tree was constructed by the “neighbour-joining” method and was bootstrapped 10000 times. 0.1 indicates the genetic distance.

**Figure 3.** Sea bream TcR $\beta$  basal expression in different tissues. TcR $\beta$  mRNA levels were expressed as a ratio relative to  $\beta$ -actin levels in the same samples after real-time PCR analysis using the tissue with the lowest expression (head kidney) as calibrator.

**Figure 4.** *In vitro* sea bream TcR $\beta$  expression analysis. LPS: TcR $\beta$  mRNA levels expressed as a ratio relative to  $\beta$ -actin levels in the same samples after real-time PCR analysis of HK leucocytes stimulated with PBS (control) and with 5  $\mu$ g/ml LPS for 4 and 24 h and normalised against the non-stimulated controls. PHA: TcR $\beta$  mRNA levels expressed as

a ratio relative to  $\beta$ -actin levels in the same samples after real-time PCR analysis of HK leucocytes stimulated with PBS (control) and with 1  $\mu\text{g/ml}$  PHA-L for 4 and 24 h and normalised against the non-stimulated controls. Controls for 4 and 24 h of incubation with PBS only are also shown in the graphs. Data were expressed as the mean  $\pm$  SD and asterisks indicates when  $p < 0.05$  with respect to the time 0 control.

**Figure 5.** 3D model of *Sparus aurata* TcR $\beta$  clone 3. The backbone ribbon and secondary structure topology are shown: yellow arrows represent  $\beta$ -strands. Amino and carboxy terminal ends are indicated. Green and yellow sticks evidenced the presence of a putative intrachain disulfide bond.

**Figure 6.** 3D model of sea bream class I MHC. The backbone ribbon and secondary structure topology are shown: yellow arrows represent  $\beta$ -strands and red cylinders represent  $\alpha$ -helices. Green and yellow sticks indicate the presence of two putative intrachain disulfide bonds.

**Figure 7.** The bars represent the binding energies (A) and the interaction energies (B) (expressed in kcal/mol) evaluated for each simulated sea bream MHC/TCR $\beta$  complex and for the experimental human and murine complexes.

#### TABLE CAPTIONS

**Table I.** Analysis of the MHC/TcR $\beta$  complexes in terms of interface surface area, intrachain H-bonds and number of interaction residues.

Figure 1

	<	signal peptide	><	variable region	
Spauc11	-MILIFLSIH	LNNIL----	SGLSLNDQVH	QTPADMFKRP	GGEAKINC <sup>CFH</sup> TI-QSYNRIL WYKQTNE--Q LQFLGYMNNIN
Spauc12	-.SSVMRIFG	.LL.C-----	F YSQVSSVTFO	EF.LQIVNES	S-.VQ.Q.S. DD-STKTQM. .FQ.KKDSL <sup>V</sup> .TLI..VYGQ
Spauc13	-.SSLMRIFG	.LL.C-----	F YSHVTAITFO	EF.PQIVSES	S-.VH.Q.S. DD-TGKLQM. .FQ.KKDSL <sup>V</sup> .TLI.FVYGQ
Spauc14	MIRN.YRIT-	.LLLW----	L .CR.QSV..D	.G.SSILGS.	N.S.T.S.N. SH-SLF.V.. .Q.PTGDAG .KLI.LTQYT
Spauc15	-.TAGLITLS	AALLC----	I I..IDGSD.T	..LLWMMEG	Q-S.TM..S. .KGT..YEMY .R.LPG-QR MKEIVFTTQS
Spauc16	-----	-----	SE...Q	...F...	..SN.DK...--
Gadmo	-.CQFIC.LG	IIFVC-----	F THEVQSVIIK	.SS.KIVRKG	AKGIQ.D.S. DD-S..PLMY .QRKD.SPS .TLI.FGYES
Stepa	-.KHVLIITG	.CFTFNII <sup>L</sup>	.S.S.S.K.D	.A.T.IYGKQ	.ET.E.T.S. K.-DN.... .L.--RN .....L...
Parol	-.PSLNTLT	FFVLW----	A A.V.HSVLIT	.W.HYISRF.	S.S.EMH.YQ ND-TD.PYMY .R.QRG-KE P.LVV.LAGS
Oncmy	-.R.LI..T	MGYRA---WA	A.S.PSN...	.G...LY.NQ	.EL..ME.S. S.-ST..V.. .S.Y-RE .V....QLK
Anapl	-----MGMW	TAWCVATFFF	GAR--AKIT	.S-SLVLKE	D...TLK.SQ ND--NH.YMS .L.QPG-KG .L.Y.SIGA
Ratno	-----	-----	MEAA.T	.S.RNRVAVT	..VTLS.RQ .N--NH <sup>D</sup> MY .R.NMG-DE .RLIH.SYDV
Musmu	-----MGSR	.FFVV-LILL	CAKHMEAA.T	.S.RSKVAVT	..KVTL <sup>S</sup> .HQ .N--NH <sup>D</sup> MY .R.DTG-HG .RLIH.SYVA
Homsa	-----MT.R	.LCYMGFYPL	GAGLMEAD <sup>IY</sup>	..RYLVIGT	.KKITL <sup>S</sup> .SQ .M--GHDKMY .Q.DPG-ME .HLIH.SYGV
				*	*
	<<	diversity and joining region	>>		
Spauc11	NGYPENGAG-	-VKIEGNANT	D <sup>Q</sup> NCTLTIEE	LKLS-SSAVY	FCAART---- --GEYEAYFG PGTKLTVLGK DDKITPPT-V
Spauc12	GSPTYE.EM-	EKQFKLTKEG	TTKG..I.HS	A...-D...	...TD---- -NNNQP... Q.....-
Spauc13	GSPTYE.EM-	EKQFKLTKEE	TTKG..I.RR	A...-D...	...KA---G INQANP... Q.....-
Spauc14	-SPTLEEPF-	KEHKVVTGDG	SSKSE.HVQ.	.R.PED.GM.	Y...PG---- --G..PI.. G.....V..
Spauc15	PPHQYESGFS	TE.FPATKKD	ALTGS..V.R	.L.N-D.G..	...VTS---- -G.NT..H.. G.....-
Spauc16	-----	-----	V..G.KK	...N....	L...R---- --TGR... G.....-
Gadmo	STQNYEDRF-	EERLNITRES	VLQG..VLT	AAE.-D...	...SMG-- EG.SEP.F.. K.....EP GCIVS....
Stepa	K...D.VDV	--T.D.D..K	GR.....NS	.SV.-....	...SYGTG- GPQTEP... K.....ET .RTV...K.
Parol	SANF.E.FK-	-SGF.AEIVQ	KKKWS.K.PS	IQEK-DE...	L...SH---- -RNTQP... Q.....EP GQAVKS.I-
Oncmy	T.F..V.FD-	---D..A	GGTS...KQ	.TPN-....	Y...---TG- TKNYNP.F.. A.....DP NI.V.E.-.
Anapl	DQEA <sup>V</sup> GDTH-	-PGYKATRLN	LSDFH.V.KP	V.MN-H.D.	...SSP--- NR.SNTQ... E...I...E. N.V.K..A-
Ratno	.RTEKGDVS-	-SGYKASRPS	QEDFS.IL.S	AS..-QT...	...S.D--- TRNT-.VF.. K..R...VED LKTV...K-
Musmu	DSTEKG <sup>D</sup> IP-	-DGYKASRPS	QE.FS.IL.L	AS..-QT...	...SSE--- NR.A-.QF.. .R....ED LRNV...K-
Homsa	.STEKG <sup>D</sup> LS-	-SESTVSRIR	TEHFP..L.S	ARP...EL <sup>S</sup> Q.	L...SSV--- GGDQ-PQH.. D..R.SI.ED LN.VF...E-
			*	**	** **
			constant Ig domain		
Spauc11	KVLEPSEKEC	RNKVEKEKRK	KTLLCVISRF	YPDHVN <sup>V</sup> TWK	INN----- -EEMSKGVAT DNMPAQ <sup>P</sup> --- NDGK <sup>F</sup> YKITS
Spauc12	.....	.....	.....	.....	-----
Spauc13	.....	.....	.....	.....	-----
Spauc14	.....	.....	.....	.....	-----
Spauc15	.....	.....	.....	.....	-----
Spauc16	.....	.....	.....	.....	-----
Gadmo	V..P...A...	.DR--QL.	.V..A.G.	...G.S.T	V.G----- -QSVI...S .HPALRV--- D.K--Q..
Stepa	.IFP.A.A...	..K-DDI..	.V..A.G.	...S.S.E	K.GKVVPDSE AKDRQEKYGV ATDS.AK-- RV.E..R..
Parol	.FR..S...S	..PIDN.RE.	..V..A.D.	...S.Y.Q	.IQLNVTSG- -VNVTR..T. .EAALR---- -KD.V.T...
Oncmy	..A.A.A...	EDR--NK.K.	..V..AT..	...T.F.Q	V..----- -VNRTE.AG. .RALWD--- K..-L.S...
Anapl	AIFS..KQ.I	QE-----S	A..V.LA.G.	...TL.LV..	V.G----- -A.RTE..G. .ETSTS--- -YENT.SL..
Ratno	SLF...A.I	AD-----Q.	A..V.LARG.	F...ELS.W	V.G----- -K.IRN..S. .PQAYK---E SNNIT.CLS.
Musmu	SLF...KA.I	A..-----Q.	A..V.LARG.	F...ELS.W	V.G----- -K.VHS..S. .PQAYK---E SN-YS.CLS.
Homsa	A.F...A.I	SHT-----Q.	A..V.LATG.	F...EL <sup>S</sup> Q.	V.G----- -K.VHS..S. .PQ.LKEQPA LND <sup>S</sup> R.CLS.
	** *	*	** *	*	** *
				<<	CPS
Spauc11	RLKVDANKWF	DPENE <sup>F</sup> KCIA	SFFN <sup>GT</sup> GT <sup>TY</sup>	HEN <sup>GT</sup> RG---	-----IEAPK TGQNITTEAY LKRSQTAKLS YGVLI <sup>I</sup> KGCV
Spauc12	.....	.....	.....	-----	-----
Spauc13	.....	.....	.....	-----	-----
Spauc14	.....	.....	.....	-----	-----
Spauc15	.....	.....	.....	-----	-----
Spauc16	.....	.....	.....	-----	-----
Gadmo	.R.E.R..Y	TGG.I.T.NV	.Y...ND.I	TSAEVY.GGD	VRW--KTEP D.E--R.EF V.VT..... .I.M.V.NI
Stepa	.R.P.AHYN	T.G.T.T.V	.Y...QNV-	LRHASID---	--S--KGES E.G-M.R.K. .HT.S..... .V.S.I
Parol	...S.ED.Y	K..WN.E..V	R....HD..	.KDSIS----	-----EQ GPDIL.R.K. .RITRQ.... .S.....SS.
Oncmy	.R.P..E.H	K...R.T..V	.YD..DNIR	VT.D.ISG--	--D--LQGS G.E-...DY. V.ST....A .SIF.A.STF
Anapl	..RISSQ...	N.L.R.E.V.	N..KNG----	-----TQ	ESIHRFIYGD A.CI.FK.N. QRSATAG.FL .IM..L.SIL
Ratno	..R.S.PF.H	N.R.H.R.QV	Q.YGL.EEDN	WSEDS <sup>P</sup> KPVT	QNISAEAWGR ADCG..SAS. QQGVLS.TIL .EI..G.ATL
Musmu	..R.S.TF.H	N.R.H.R.QV	Q.YGLSEEDK	WPE.SPKPVT	QNISAEAWGR ADCG..SAS. HQGVLS.TIL .EI.LG.ATL
Homsa	..R.S.TF.Q	N.R.H.R.QV	Q.YGLSENDE	WTQDRAPVT	QIVSAEAWGR ADCGF.SVS. QQGVLS.TIL .EI.LG.ATL
	**	*	*	*	*
	<<	CYT	>		TM
Spauc11	YGAFVMFLVW	KLPGSSGKRN	N--		
Spauc12	.....	.....	--		
Spauc13	.....	.....	--		
Spauc14	.....	.....	--		
Spauc15	.....	.....	--		
Spauc16	.....	.....	--		
Gadmo	..V..TI.A.	..GLGRSHAT	AKK		
Stepa	...IG....	..Q....H.	--		
Parol	....A...R	.QS.TE.Q.	---		
Oncmy	..LV..VMI.	.FQ...E.QI	---		
Anapl	..I...GMML	RSK-----	---		
Ratno	.AVL.ST..V	MAMVKRKSS-	---		
Musmu	.AVL.SG..L	MAMVKKKNS-	---		
Homsa	.AVL.SA..L	MAMVKRKDF-	---		
	*				

Figure 2

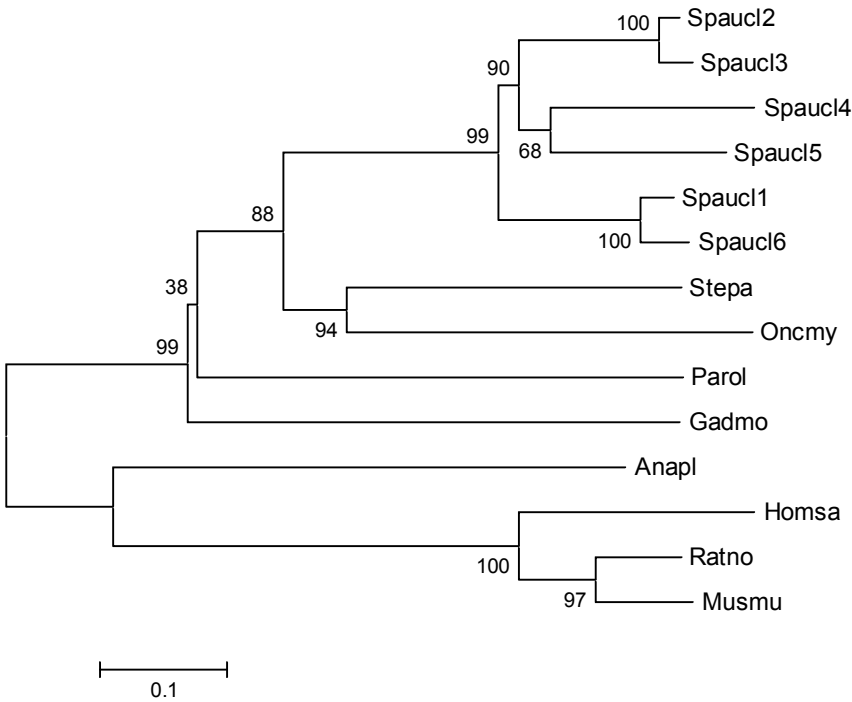




Figure 3

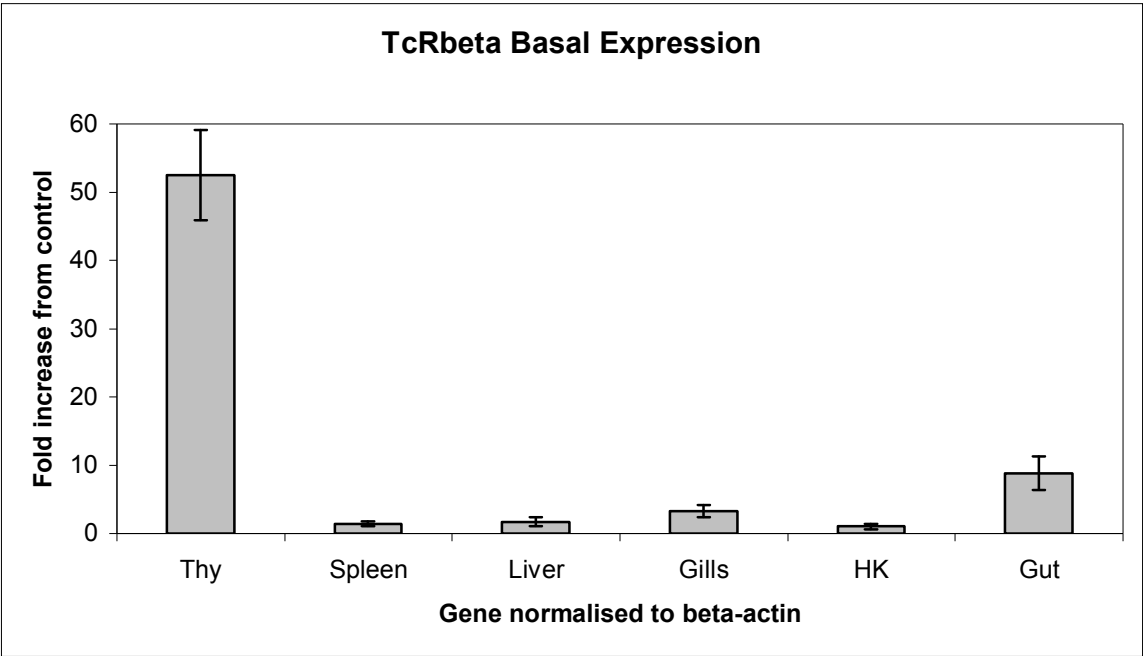


Figure 4

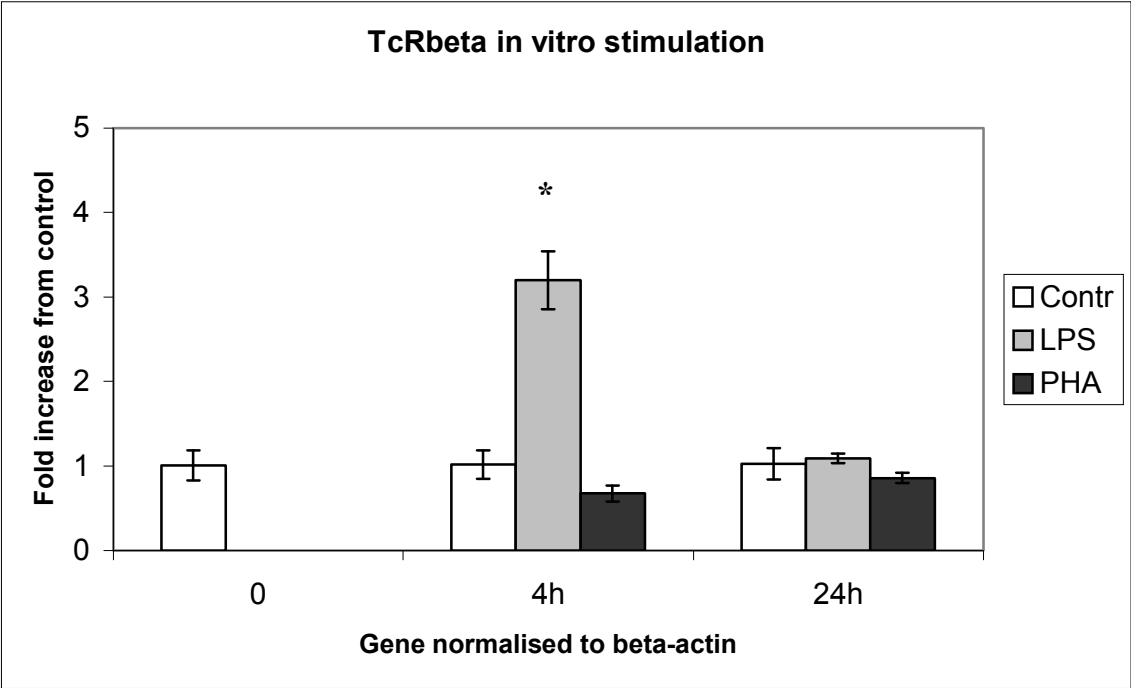


Figure 5

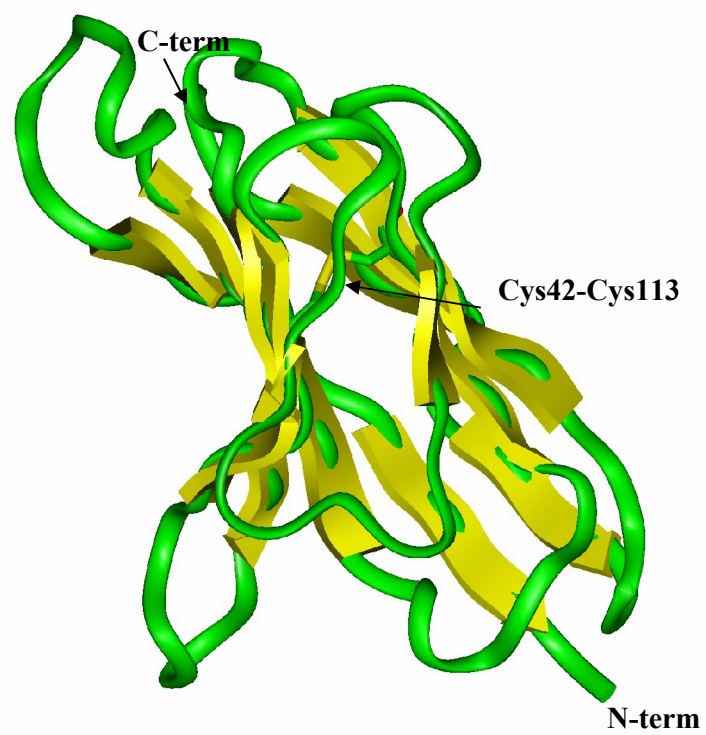


Figure 6

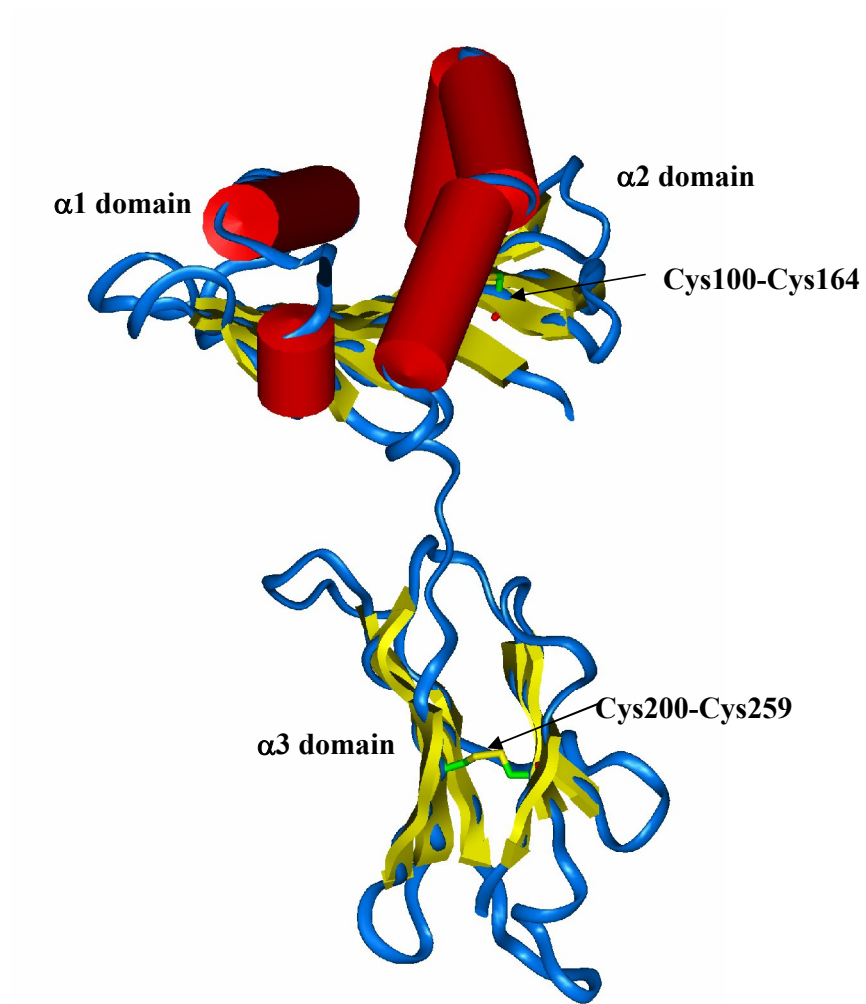


Figure 7

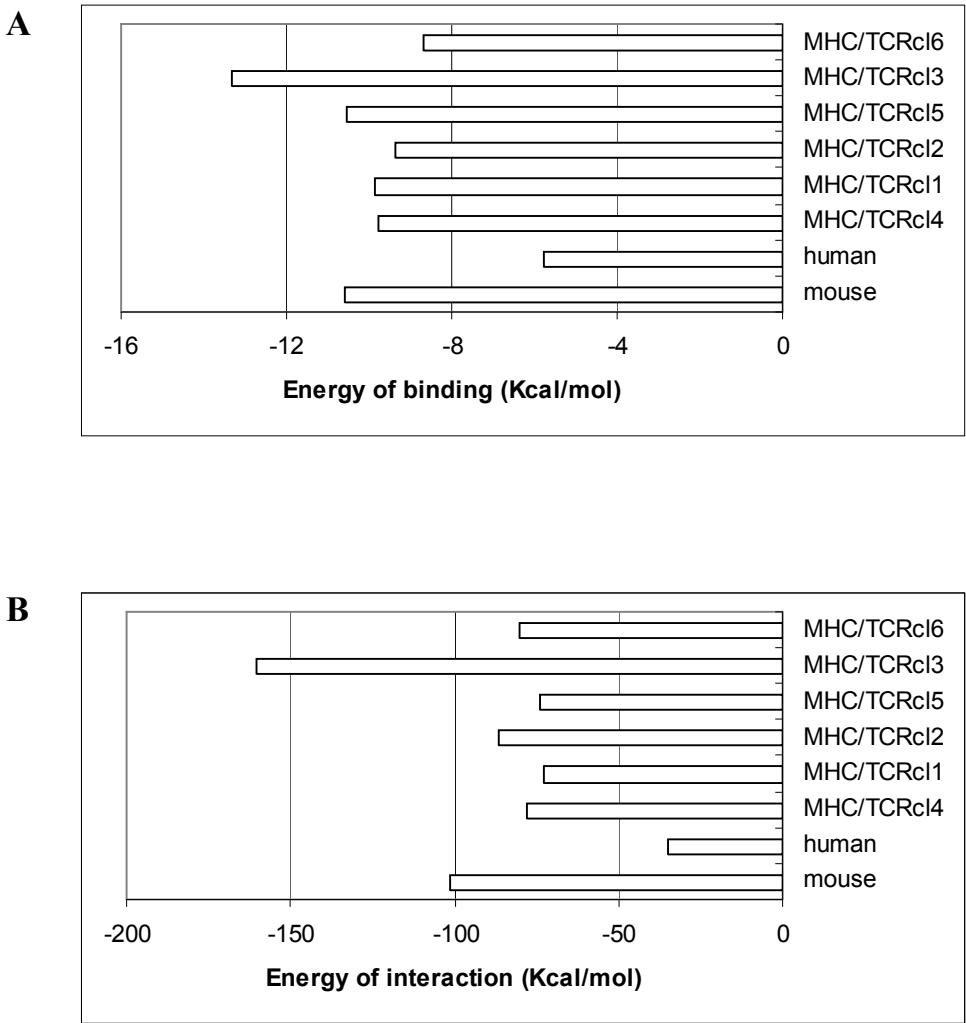


Table I

	Interface Surface Area	Intrachain H-bonds	Interaction residues
MHC murine	490.06	3	15
TcRβ murine	500.53	3	18
MHC human	262.30	2	10
TcRβ human	300.76	2	9
MHC	642.91	3	18
TcRβ clone 4	649.22	3	16
MHC	672.71	2	19
TcRβ clone 1	670.04	2	18
MHC	726.4	6	19
TcRβ clone 2	682.4	6	19
MHC	622.88	4	18
TcRβ clone 5	640.17	4	18
MHC	1056.8	11	18
TcRβ clone 3	997	11	18
MHC	618.35	5	21
TcRβ clone 6	608.93	5	17